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Telomere-Associated Protein TIN2 Is Essential for Early Embryonic Development through a Telomerase-Independent Pathway

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TIN2 is a negative regulator of telomere elongation that interacts with telomeric DNA repeat binding factor 1 (TRF1) and affects telomere length by a telomerase-dependent mechanism. Here we show that inactivation of the mouse TRF1-interacting protein 2 (TIN2) gene results in early embryonic lethality. We further observed that the embryonic lethality of TIN2 mutant mice was not affected by inactivation of the telomerase reverse transcriptase gene, indicating that embryonic lethality is not the result of telomerase-dependent changes in telomere length or function. Our findings suggest that TIN2 has a role independent of telomere length regulation that is essential for embryonic development and cell viability.

Fn* Telomeres are complex structures at chromosome ends that function to prevent chromosome fusions and genomic instability (6). Mammalian telomeres consist of double-stranded TT AGGG repeats and associated proteins. A number of these telomere-associated proteins have been identified, including telomeric repeat binding factors 1 and 2 (TRF1 and TRF2) (5), TRF1-interacting protein 2 (TIN2) (12), tankyrase 1 (19) and 2 (9), protein interacting with NIMA-interacting factor 1 (22), ATM (13), Ku (8), Rap1 (14), DNA-PK (7), and POT1 (1, 15). TRF1 is a ubiquitously expressed protein that binds to double-stranded telomeric DNA repeats (2, 18). The acidic amino terminus of TRF1 contains a conserved homodimerization motif, and the carboxyl terminus of TRF1 has a DNA-binding domain homologous to Myb oncoprotein (4, 5). TRF1 acts as a negative regulator of telomere length maintenance, with overexpression of TRF1 resulting in telomere shortening and expression of dominant-negative (DN) TRF1 causing telomerase-dependent telomere elongation (2, 21).

TIN2 is a protein that interacts directly with TRF1 and is an essential mediator of TRF1 activity (11, 12). In the presence of telomerase activity, overexpression of TIN2 inhibits telomere elongation in human cell lines, whereas expression of DN N-terminal deletion mutants of TIN2 enhances telomere elongation (12). The TIN2 protein contains N-terminal basic and acidic regions, a central TRF1-binding domain, and a C-terminal region. The basic and acidic regions are required for the regulation of TRF1 activity. The TRF1-binding domain of TIN2 associates with the TRF1 homodimerization domain, providing for the recruitment of TIN2 to the telomere (12). Kim et al. (11) have suggested that binding of wild-type TIN2 induces changes in TRF1 conformation that in turn favor a telomeric structure which is inaccessible to telomerase, thus preventing telomerase-mediated telomere elongation. The absence of TIN2 would conversely favor telomerase accessibility and telomere elongation.

The physiological role of TIN2 during in vivo development and in normal cell function has not previously been assessed. To better understand the in vivo function of TIN2, we have therefore studied the effect of TIN2 mutation on mouse development by using gene targeting technology. We found that TIN2 deletion results in early embryonic lethality through a mechanism that is independent of telomerase function.

MATERIALS AND METHODS

Targeting vector, electroporation, and selection. For the TIN2 gene, a replacement type targeting vector was constructed from an 8.0-kb 129/Sv (Stratagene) mouse genomic fragment containing the TIN2 coding sequence (Fig. 1A). F1 The *neo* gene with the phosphoglycerate kinase 1 promoter and the bovine growth hormone polyadenylation sequence (pGKneobpA) was employed as a positive selectable marker; the pGK-thymidine kinase cassette was used as a negative selectable marker. Electroporation and selection were performed with the CJ7 embryonic stem (ES) cell line as described elsewhere (20). DNA from G418- and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU)-resistant ES clones was screened by diagnostic ApaI restriction enzyme digestion AQ: A and with a 3′ probe external to the targeting vector sequence, as shown in Fig. 1A. Recombinant clones containing the predicted 13-kb rearranged band were obtained at a frequency of 1/15.

Generation of mutant mice. Two independent ES cell clones targeted for the TIN2 recombination were injected into C57BL/6 blastocysts and generated chimeras that transmitted the mutated allele to progeny (3). Mice were bred in a specific-pathogen-free facility with food and water ad libitum.

Mice. TIN2 heterozygotes were maintained on a *Mus musculus* 129 background or backcrossed to an *M. musculus* C57BL/6 (B6) background. $TIN2^{+/-}$ $TERT^{-/-}$ mice were generated by breeding $TIN2^{+/-}$ mice with $TERT^{-/-}$ mice. The generation and characterization of mTERT knockout mice is described elsewhere (●●●. Chiang et al., submitted for publication). All animals were AQ: B housed at Bioqual (Rockville, Md.).

DNA isolation. ES cells and mouse tail preparations were lysed in 500 μl of DNA lysis buffer containing 100 mM Tris-HCl (pH 8.0), 200 mM NaCl, 10 mM EDTA, 2% sodium dodecyl sulfate, and 50 μg of proteinase K at 55°C overnight. Lysates were extracted with 500 μl of phenol. DNA was collected from the upper phase by ethanol precipitation and centrifugation at 12,000 rpm and dissolved in AQ: C 200 μl of Tris-EDTA buffer.

Southern blot analysis. For Southern blot analysis, 50 μg of DNA was digested with ApaI, electrophoresed on a 0.7% agarose gel, transferred to a nylon membrane, and probed with probe A (Fig. 1B).

PCR genotyping. DNA was prepared as described above, and PCR was performed. Oligonucleotides used for genotyping of TIN2 were NEO1 (5′-TTC

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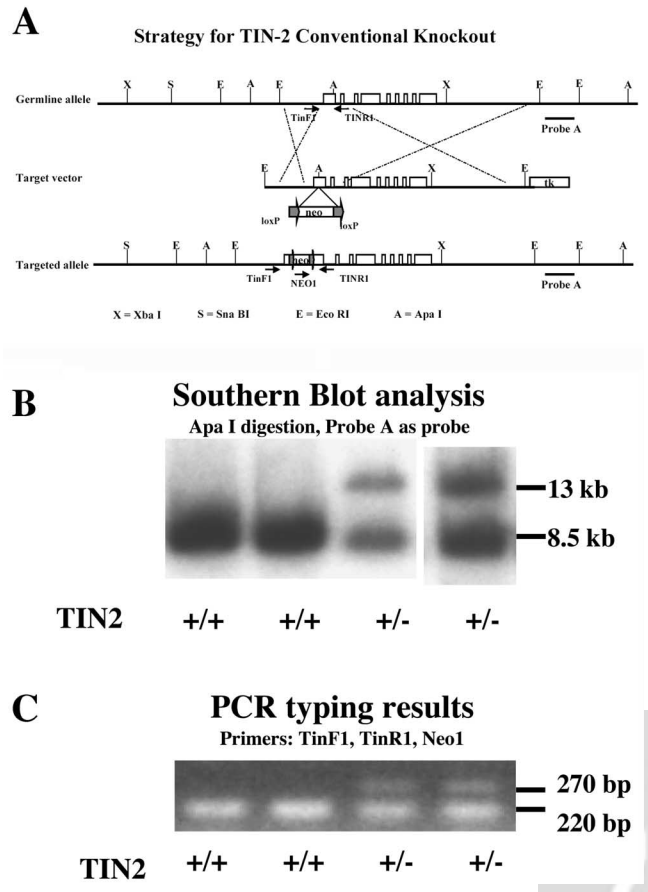

FIG. 1. Generation of TIN2-deficient mice by gene targeting. (A) Gene targeting strategy and restriction map of the gene. The open boxes represent the exons of the TIN2 gene. A neomycin resistance gene was inserted into exon 1 5′ of the translation initiation codon ATG. (B) Southern blot analysis of mouse tail DNA. The 8.5- and 13-kb bands represent the germ line and targeted alleles, respectively. (C) PCR analysis for the TIN2 genotype. The 220- and 270-bp PCR products represent the wild-type and targeted alleles, respectively.

TGG ATT CAT CGA CTG TG-3′), TINF1 (5′-TAC GTA CTC TTG CGT TTC CCT C-3′), and TINR1 (5′-TTC TCT CAT CTG CAC CGG AAG G-3′). The amplification reaction was carried out in a 25-μl volume containing 1 to 100 ng of DNA, 25 pmol of each oligonucleotide, and 12.5 μl of Supermix buffer (Invitrogen). PCR conditions were 35 rounds of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min. PCR products were revealed by agarose gel electrophoresis and ethidium bromide staining. The 220-bp band corresponds to the wild type, and the 270-bp band corresponds to knockout TIN2 (Fig. 1C).

Genotyping of embryos at day 7.5 and 10.5. Timed pregnant mice were prepared by intercrossing $TIN2^{+/-}$ mice. Embryos were isolated, digested, and genotyped by PCR as described above.

In vitro culture and genotyping of blastocysts. Three- to four-day-old embryos from $TIN2^{+/-}$ crosses were isolated and cultured at 37°C in ES cell RPMI medium containing 15% fetal bovine serum and 100 U of LIF/ml (16, 17). Cells were photographed and then digested in 100 μl of DNA lysis buffer as described above. DNA was precipitated, centrifuged, and dissolved in 10 μl of H_2O. Genotyping was carried out as described above except that PCR conditions were 5 rounds of 95°C for 30 s, 49°C for 30 s, and 72°C for 1 min; 5 rounds of 95°C for 30 s, 52°C for 30 s, and 72°C for 1 min; 5 rounds of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and 35 rounds of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min.

RESULTS

Disruption of the TIN2 gene results in early embryonic lethality. In vitro studies have demonstrated that TIN2 interacts with TRF1 and is an essential mediator of TRF1 activity in telomerase-dependent telomere length regulation. However, little is known about the in vivo function of TIN2 in mammalian cells and during development. To better understand the physiological roles of TIN2, mouse gene targeting technology was used. It has been shown that the B6 mouse TIN2 gene is located on chromosome 14 (GenBank), is approximately 3.98 kb in length, and consists of 9 exons. Before 129/SvJ strain mouse genomic DNA was used to make a gene targeting construct, the TIN2 gene was mapped with 12 common DNA restriction enzymes. The mapping results showed that the 129/SvJ strain TIN2 gene has a gene structure similar to that of B6 (data not shown). A TIN2 gene targeting vector was constructed as shown in Fig. 1A. A neomycin resistance gene was inserted into the first exon of TIN2 upstream of the ATG translation initiation codon. To screen for targeting of the vector to the TIN2 locus in ES cells, ES cell DNA was digested with ApaI and blotted with probe A as shown in Fig. 1B. The 8.5-kb DNA fragment was the TIN2 germ line allele, and the 13-kb fragment represented the targeted allele. Eleven targeted ES cells were identified in 168 clones screened. Two targeted ES clones were used to make chimeric mice. Heterozygous $TIN2^{+/-}$ mice were identified by Southern blot analysis or PCR from the offspring of chimeric mice (Fig. 1B and C). The 220-bp PCR product corresponded to the wild-type allele, and the 270-bp product corresponded to the targeted allele of TIN2 (Fig. 1C).

TABLE 1. There are no $TIN2^{-/-}$ offspring from $TIN2^{+/-}$ crosses AQ: D

Result	No. of $TIN2^{+/-} \times TIN2^{+/-}$ offspring		
	$TIN2^{+/+}$	$TIN2^{+/-}$	$TIN2^{-/-}$
Expected	59	118	59
Observed	80	157	0

No homozygous $TIN2^{-/-}$ mice were obtained among 237 offspring of intercrosses of $TIN2^{+/-}$ mice as shown in Table 1. T1 This finding indicated that TIN2 is essential for mouse development and that homozygous inactivation of TIN2 is therefore lethal. The ratio of $TIN2^{+/-}$ to $TIN2^{+/+}$ offspring was approximately 2 to 1, suggesting that there is no haploinsufficiency and decreased viability in $TIN2^{+/-}$ mice.

To determine the time of death during development of $TIN2^{-/-}$ embryos, embryos were isolated from TIN2 heterozygous intercrosses at embryonic day 7.5 and 10.5, and individual embryos were genotyped by PCR or Southern blot analysis. No homozygous TIN2-deficient embryos were found in either day 7.5 litters or day 10.5 litters (Table 2). Thus, TIN2-deficient T2 embryos die before day 7.5 of their embryonic development.

TABLE 2. Day 3.5, but not day 7.5 or 10, $TIN2^{-/-}$ embryos were found from $TIN2^{+/-}$ crosses AQ: E

Embryonic day	No. of embryos		
	$TIN2^{+/+}$	$TIN2^{+/-}$	$TIN2^{-/-}$
10.5	7	17	0
7.5	9	15	0
13.5	7	22	9

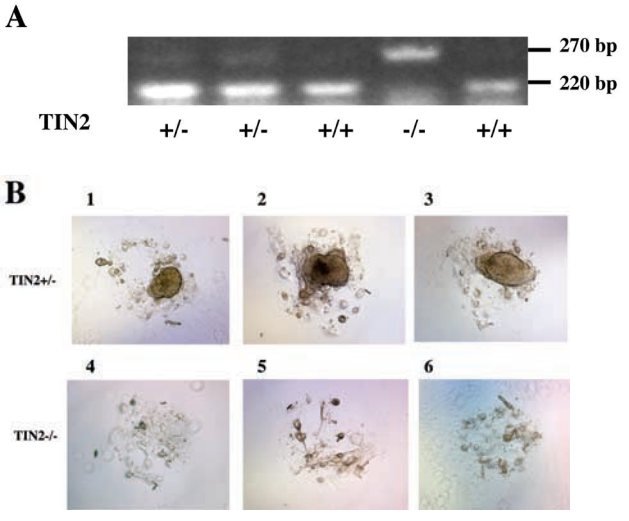

FIG. 2. (A) PCR genotyping of 3.5-day-old TIN2 embryos. The 220- and 270-bp PCR products represent the wild-type and targeted alleles, respectively. (B) In vitro-cultured 3.5-day-old embryo in ES cell medium. Pictures in the upper panel represent individual $TIN2^{+/-}$ embryos after 5 days of culture. Pictures in the lower panel represent individual $TIN2^{-/-}$ embryos after 5 days of culture.

ES cells have a growth and/or survival defect during in vitro culture of TIN2-deficient blastocysts. ES cells can be derived from blastocysts, and TIN2-deficient ES cell lines could thus be established for further study of TIN2 function during the earliest stages of embryonic development. Day 3.5 embryos were isolated from $TIN2^{+/-}$ intercrosses and cultured in ES cell culture medium. All embryos were of similar appearance and viability at the initiation of culture. Cells were collected and genotyped by PCR after 5 days of culture (Fig. 2A). Approximately one-fourth of ES cell cultures were identified as homozygous $TIN2^{-/-}$, indicating that homozygous knockouts survive to day 3.5 in the expected proportions (Table 2). However, it was striking that $TIN2^{-/-}$ cultures were uniformly defective in their survival and differentiation in comparison to wild-type or $TIN2^{+/-}$ ES cells. Whereas wild-type ES cell clones grew to form multilayered cell masses, $TIN2^{-/-}$ ES cell cultures were flat and cell numbers were much lower than those of wild-type or $TIN2^{+/-}$ clones (Fig. 2B). These differences in culture were observed in a masked fashion, since genotype was determined only after completion of culture, and were consistent for 9 $TIN2^{-/-}$ cultures and 29 $TIN2^{+/+}$ or $TIN2^{+/-}$ cultures. A growth and/or survival defect was thus apparent in $TIN2^{-/-}$ cells at an early stage of ES cell culture. It should be noted that TIN2 protein may be essential even in the first days of embryogenesis, where this requirement could be obscured by the persistence of maternal egg-derived mRNA or protein.

Embryonic lethality of TIN2 deficiency is not telomerase dependent. The known function of TIN2 involves its interaction with TRF1 to down-regulate the telomerase-dependent elongation of telomeres. It therefore appeared possible that embryonic lethality in TIN2 knockout mice may be mediated by telomerase-dependent effects. To explore this possibility, $TIN2^{+/-}$ mice were bred to mTERT-deficient mice that lack telomerase activity. These telomerase-deficient mice have normal embryonic development during their early generations and manifest the effects of telomere dysfunction only after progressive telomere shortening occurs through the breeding of multiple generations with homozygous telomerase deficiency. Intercrosses were carried out between $TIN2^{+/-}$ $TERT^{-/-}$ mice which were either early (G_1) or late (G_5)-generation mTERT homozygous knockouts. As shown in Table 3, neither $TIN2^{-/-}$ $TERT^{-/-}$ (G_1) nor $TIN2^{-/-}$ $TERT^{-/-}$ (G_5) offspring were observed. Thus, the embryonic lethality of TIN2 deficiency occurs in early and late-generation telomerase-deficient genetic backgrounds, indicating that the requirement for TIN2 in mouse development is telomerase independent.

TABLE 3. There are no $TIN2^{-/-}$ offspring from $TIN2^{+/-}$ $TERT^{-/-}$ crosses

Growth stage	No. of $TIN2^{+/-}$ $TERT^{-/-}$ × $TIN2^{+/-}$ $TERT^{-/-}$ offspring		
	$TIN2^{+/+}$	$TIN2^{+/-}$	$TIN2^{-/-}$
G_1	8	18	0
G_5	19	31	0

DISCUSSION

TIN2 has been identified as a TRF1 binding protein that is essential for the negative regulatory function of TRF1 in modulating telomerase-dependent telomere elongation (12). In the present study, a genetic approach was used to examine the in vivo function of TIN2. We found that TIN2 deficiency in mice resulted in early embryonic lethality in genetic backgrounds with either intact or absent telomerase activity. These findings indicate that TIN2 is essential in embryonic development and demonstrate a previously undescribed telomerase-independent function of TIN2.

TIN2 and TRF1 appear to act as a complex to mediate telomere length regulation. TRF1 interacts with TIN2 through the TRF1 dimerization domain and the TRF1-binding region of TIN2 (11). Overexpression of either TRF1 or TIN2 leads to telomere shortening, whereas expression of either DN TRF1 or DN TIN2 results in telomerase-dependent telomere elongation (12). It has been proposed that TIN2 functions in this role by binding to TRF1 dimers and altering the conformation of TRF1 (11). This alteration favors the association of TRF1 with telomeric DNA repeats, which in turn interferes with the accessibility of telomerase to telomere ends. It may therefore be predicted that inactivation of either TRF1 or TIN2 would result in elongation of telomeres in vivo. To test this prediction, TIN2 knockout mice were generated in the present study. However, TIN2 inactivation resulted in early embryonic lethality during mouse development, precluding analysis of the effect of TIN2 on telomere length regulation. Similarly, the rapid death of $TIN2^{-/-}$ ES cells in culture has prevented analysis of the mechanism mediating lethality. Recently, Karlseder et al. (10) reported that inactivation of the mouse TRF1 gene results in embryonic lethality and that TRF1 knockout blastocysts have a cell growth defect and increased apoptosis. The phenotype of TIN2 knockout mice thus appears to be similar to that of TRF1-deficient mice. To understand the roles of TIN2 and TRF1 in embryonic development and in adult animals, studies

of inducible TIN2 or TRF1 conditional knockout mice will be informative. Inducible inactivation of TIN2 or TRF1 will allow determination of whether defects in telomere length regulation, apoptosis, or cell cycle progression result from gene inactivation and contribute to embryonic lethality in TIN2-deficient mice. We have, in fact, generated TIN2 conditional knockout constructs by using *cre*/*loxP* techniques and will use these constructs in studies of inducible and tissue-specific TIN2 inactivation.

Our studies have indicated that telomerase inactivation does not reverse the embryonic lethality of TIN2 deficiency. This demonstrates that TIN2 has a telomerase-independent function that is required during mouse development. These observations imply that, in addition to the telomerase-dependent functions played by TIN2-TRF1 complexes, both TIN2 and TRF1 also function in telomerase-independent roles. Additional telomere-associated proteins may be involved in the telomerase-independent function of TIN2 and TRF1. Among these, constitutive knockouts of some proteins such as ku and ATM are not lethal and are therefore not straightforward candidates for mediation of TIN2 or TRF1 lethality. Others, such as tankyrase 1 and 2 are among the candidates that merit further study.

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AUTHOR QUERIES

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AQA—If FIAU not spelled out as meant, please correct here.

AQB—Please provide initial(s) for Chiang here. Also, please provide initials and surnames for subsequent authors.

AQC—Please give the "x g" value or the brand name of the instrument for centrifugation here.

AQD—Table 1 redrawn. Please check data carefully.

AQE—Table 2 redrawn. Please check data carefully.

AQF—Table 3 redrawn. Please check data carefully.